impurity levels are now less than 5 ppm (the first silicas used had typical impurity levels of 1000–2000 ppm). In the silica support materials the latest developments have been towards base-deactivated materials and have tended to focus on the new very pure silicas. Particle size distributions have also become tighter around given means, leading to more stable and reproducible columns.

In column design, the move has been to cartridge type systems having smaller tube i.d. with smaller particles and shorter lengths.

The focus on development is now moving to micro systems. Future developments are likely to be systems with smaller and smaller column i.d., and even complete columns on microchips.

See Colour Plates 21, 22.

See also: **II/Chromatography: Liquid:** Mechanisms: Reversed Phases. **III/Porous Graphitic Carbon: Liquid Chromatography. Porous Polymers: Liquid Chromatography**.

Further Reading

Balke ST (1984) *Quantitative Column Liquid Chromatography*. Amsterdam: Elsevier.

Horvath C (ed.) (1980) *High Performance Liquid Chromatography*, *Advances and Perspectives*, vol. 1: *History of HPLC*, *Chemically*, *Bonded Phases*, *Secondary Equilibria*, *Gradient Elution*. New York: Academic Press.

Horvath C (ed.) (1980) *High Performance Liquid Chromatography*, *Advances and Perspectives*, vol. 2: *Optimisation*, *Adsorption Chromatography*, *Reversed-Phase HPLC*. New York: Academic Press.

Kucera P (1984) *Microcolumn High Performance Liquid Chromatography*. Amsterdam: Elsevier.

Nawrocki J (1997) The silanol group and its role in liquid chromatography. *Journal of Chromatography A* 779: 29–71.

Neue UD (1997) *HPLC Columns. Theory, Technology, and Practice*. New York: Wiley-VCH.

Novotny M and Ishii D (eds) (1985) *Microcolumn Separations*. Amsterdam: Elsevier.

Scott RPW (1992) *Liquid Chromatography Column Theory*. New York: Wiley.

Scott RPW (ed.) (1984) *Small Bore Liquid Chromatography Columns*: *Their Properties and Their Uses*. New York: Wiley.

Unger KK (ed.) (1990) *Packings and Stationary Phases in Chromatographic Techniques*. New York: Marcel Dekker.

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Countercurrent Liquid Chromatography

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Introduction

Countercurrent chromatography (CCC) belongs to the family of liquid partition chromatography but with one distinct feature: the system totally eliminates the use of a solid support. Unlike liquid chromatography (LC), CCC utilizes two immiscible solvent phases. The partition process takes place in an open column where one phase (the mobile phase) continuously passes through the other (stationary phase), which is permanently retained in the column. To retain the stationary phase within the column, the system uses effective combinations of the column configuration and a force field (gravitational or centrifugal). Hence CCC instruments display a variety of forms that are quite different from those used in LC.

Because no solid support is used, CCC can eliminate all the complications arising from the use of a solid support such as adsorptive sample loss and denaturation, tailing of solute peaks and contamination.

The Two Basic CCC Systems

All existing CCC systems have been developed from two basic forms, the hydrostatic equilibrium system and the hydrodynamic equilibrium system (**Figure 1**).

The basic hydrostatic system (left) uses a stationary coiled tube. The coil is first filled with the stationary phase (either the lighter or the heavier phase of an equilibrated two-phase solvent system) and the mobile phase is introduced from one end of the coil. Owing to the action of gravity, the mobile phase percolates through the segment of the stationary phase in one side of the coil. This process continues until the mobile phase reaches the other end of the coil. Thereafter the mobile phase only displaces the same phase leaving the stationary phase in the coil. Consequently, solutes introduced at the inlet of the coil are partitioned between the two phases in each helical turn and separated according to their partition coefficients.

The basic hydrodynamic system (right) uses a similar arrangement except that the coil is rotated around its own axis. This simple motion produces a profound effect on the hydrodynamic process in the coil by generating an Archimedean screw force. All

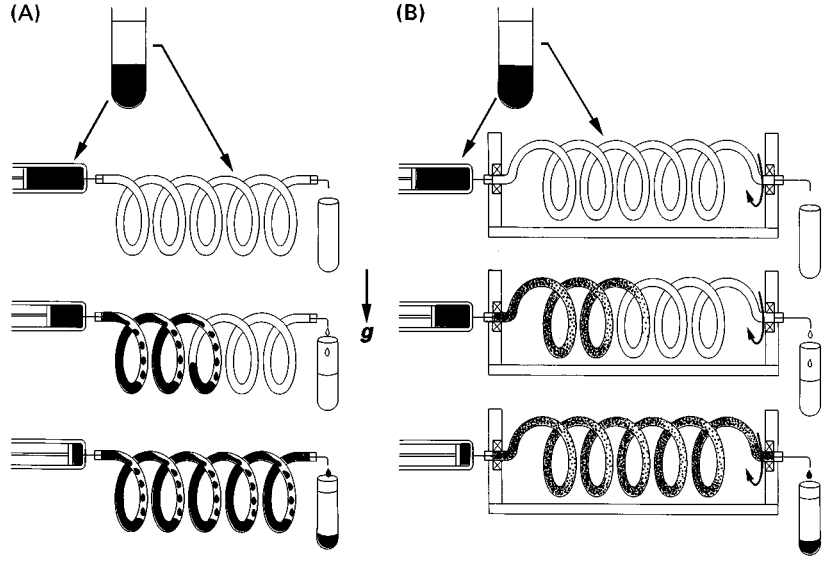

Figure 1 Two basic CCC systems. (A) Hydrostatic equilibrium system. (B) Hydrodynamic equilibrium system.

materials present in the coil that are either heavier or lighter than the suspending medium are driven toward one end of the coil. This end is conventionally called the head; the other end is called the tail.

Owing to the effect of this Archimedean screw force, the mobile phase introduced through the head end of the coil immediately interacts with the stationary phase to establish a hydrodynamic equilibrium. The two solvent phases are distributed fairly evenly in each helical turn where they are vigorously mixed by the rotation of the coil. After the entire coil reaches this equilibrium state, the mobile phase only displaces the same phase, leaving a large amount of the other phase stationary in the coil. Consequently, solutes introduced through the inlet of the coil are efficiently separated and eluted out in the order of their partition coefficients.

Each basic CCC system described above has its own merits. The hydrostatic system gives a stable retention of the stationary phase whereas the hydrodynamic system yields a higher partition efficiency by vigorous mixing of the two phases with the rotation of the coil. Several efficient CCC systems have been developed from each basic system.

Hydrostatic CCC Systems

During the early 1970s, hydrostatic CCC systems were rapidly developed because of their simplicity. Typical hydrostatic CCC systems are schematically illustrated in **Figure 2**. They are classified into gravitational and centrifugal schemes.

In the gravitational schemes, the basic hydrostatic equilibrium systems (HSES, shown in Figures 1 and 2, left) is modified as follows. One side of the coil, which is entirely occupied by the mobile phase and therefore forms an inefficient dead space, is displaced with narrow bore transfer tubes, while the other side of the coil, which provides an efficient column space, is changed to large-bore straight tubes. In droplet CCC (DCCC, Figure 2A), the vertical column is first filled with the stationary phase and the mobile phase is introduced from one end. The mobile phase then forms multiple droplets, each occupying the space across the diameter of the column, thus dividing the column into a number of partition units. The system requires use of a proper solvent system for droplet

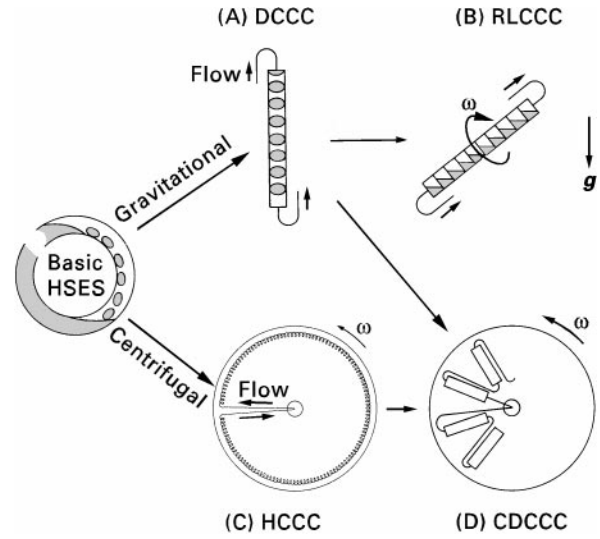

Figure 2 Development of hydrostatic CCC systems. These diagrams illustrate a variety of CCC schemes developed from the basic hydrostatic equilibrium system (HSES) shown in Figure 1, left. They are divided into the gravitational (upper) and centrifugal (lower) schemes.

formation. Rotation locular CCC (RLCCC, Figure 2B) utilizes a locular column prepared by the insertion of centrally perforated discs to divide the column space into multiple partition units called locules. This locular column is first filled with the stationary phase followed by introduction of the mobile phase at one end while the column is tilted and rotated about its own axis. When both column inclination and rotation speed are optimized, each locule holds a desired volume of the stationary phase, which is steadily mixed with the mobile phase by the rotation of the column.

In the centrifugal schemes, analytical helix CCC (HCCC) was developed by reducing the dimensions of the coil, which is then placed around the periphery of a centrifuge bowl (Figure 2C). Under a centrifugal force field the same partition process takes place in each turn of the coil as observed in the basic hydrostatic system, but with a much higher efficiency due to the reduced column dimensions. Another centrifugal scheme called centrifugal droplet CCC (CDCCC) performs droplet CCC in a centrifugal force field (Figure 2D).

Hydrodynamic CCC Systems

Rotary-Seal-Free-Flow-Through Systems

The development of the hydrodynamic CCC systems was initiated by introduction of various flow-through centrifuge systems that can perform continuous elution without the use of conventional rotary seals. A series of such centrifuge systems is schematically illustrated in **Figure 3**. The diagrams show the

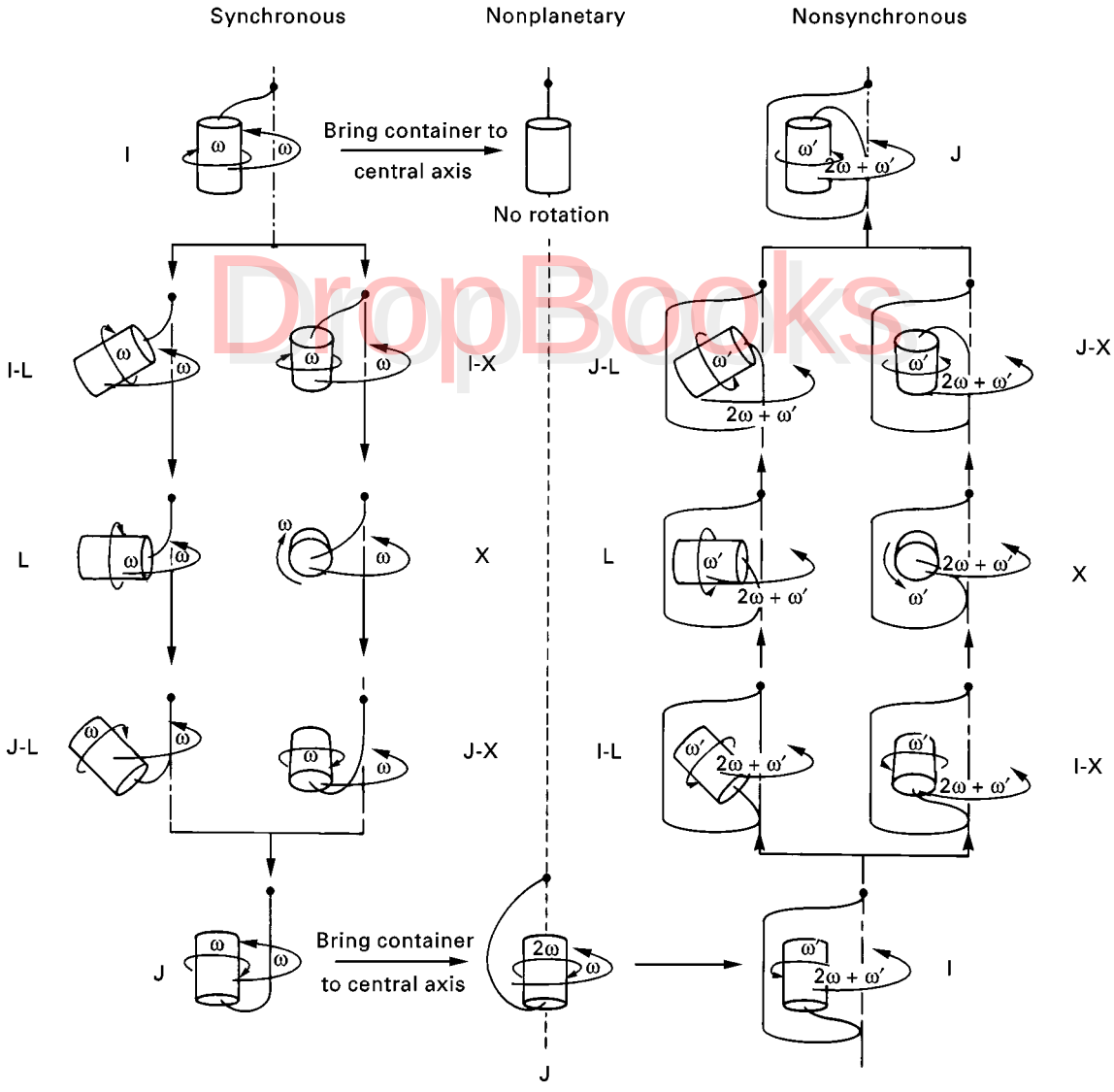

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Figure 3 A series of flow-through centrifuge systems free of rotary seals.

orientation and motion of a cyclindrical coil holder with a bundle of flow tubes, the end of which is supported on the centrifuge axis at the point marked with a black dot.

These centrifuge systems are classified in three groups according to their modes of planetary motion. The synchronous schemes (left column) produce a synchronous planetary motion of the coil holder, i.e. one rotation about its own axis during one revolution around the central axis of the centrifuge. In the nonsynchronous schemes (right column) the rates of rotation and revolution of the holder are independently adjustable. The nonplanetary scheme (middle column) produces simple rotation as in the conventional centrifuge system. In the Type I synchronous planetary motion (left, top) the holder revolves around the central axis of the centrifuge and synchronously rotates about its own axis in the opposite direction. This synchronous counterrotation of the holder steadily unwinds the twist of the tube bundle caused by revolution, thus eliminating the need for the rotary seal. This same principle can be applied to other synchronous schemes with tilted (Types I-L and I-X), horizontal (Types L and X), dipping (Types J-L and J-X) or even inverted (Type J) orientation of the holder. The Type J synchronous scheme is a transitional form to the nonplanetary scheme. When the holder of the Type J synchronous scheme is shifted to the central axis of the centrifuge, the rates of rotation (ω) and revolution (ω) of the holder are added and the holder rotates at an angular velocity of 2ω. In this case the tube bundle rotates around the holder at ω in the same direction as that of the holder to unwind the twist caused by the rotation of the holder. This nonplanetary scheme is further transformed to the nonsynchronous schemes. On the base of the nonplanetary scheme, the holder is again shifted towards the periphery of the centrifuge to perform a synchronous planetary motion. By selecting the holder orientation as specified in the synchronous schemes, the respective types of nonsynchronous schemes are produced.

Each scheme produces a specific pattern of the centrifugal force field that can be utilized for performing CCC. Among these, the Type J synchronous planetary motion is found to be most useful since it can perform high speed CCC that yields efficient separations in a short elution time.

Mechanism of High Speed CCC

The design of the Type J coil planet centrifuge is schematically illustrated in **Figure 4**. A cylindrical coil holder is equipped with a gear that is coupled to an identical stationary gear mounted at the central

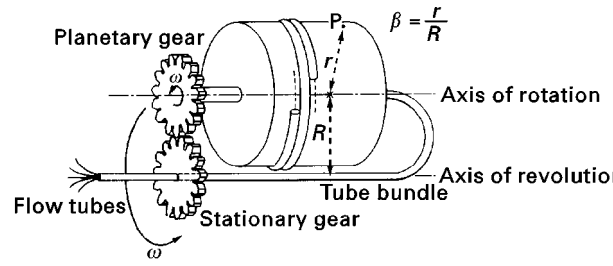

Figure 4 Design principle of Type J coil planet centrifuge.

axis of the centrifuge. This gear arrangement produces a synchronous planetary motion of the coil holder, i.e. revolution around the central axis of the centrifuge and rotation about its own axis, both at the same angular velocity and in the same direction as indicated by the arrows. As mentioned earlier, this planetary motion prevents the flow tubes from twisting and, therefore, the system permits continuous elution through the rotating column without the use of a conventional rotary seal device. The coil is directly wound around the holder as shown in the diagram. In practice, a long tube (usually over 100 m in length) is wound around a spool-shaped holder to form multiple coiled layers.

The mechanism of high speed CCC using this centrifuge design is illustrated in **Figure 5**, where all coils are shown as straight tubes for simplicity. When the coil is filled with two immiscible solvent phases and subjected to the planetary motion, the two phases are distributed in the coil in such a way that one phase (head phase) entirely occupies the head side and the other phase (tail phase) occupies the tail side (Figure 5A). This unilateral hydrodynamic distribution of

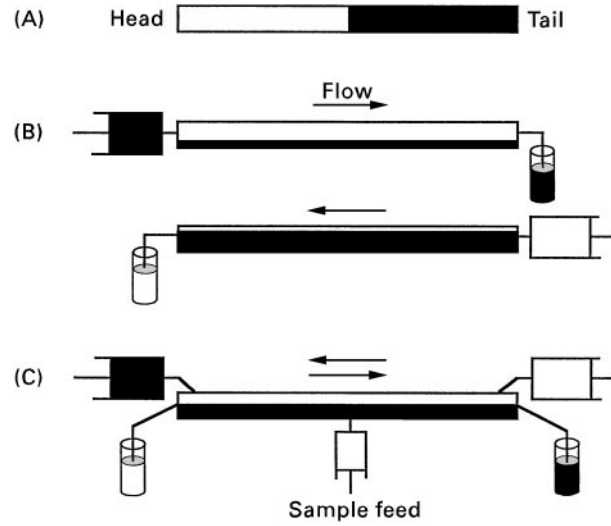

Figure 5 Mechanism of high-speed CCC. (A) Bilateral hydrodynamic equilibrium in a closed coil. (B) One-way elution modes. (C) Dual countercurrent system.

the two phases clearly indicates that the head phase (white), if introduced at the tail, would travel through the tail phase (black) toward the head, and similarly the tail phase, if introduced at the head, would travel through the head phase toward the tail. The above hydrodynamic trend can be efficiently utilized for performing CCC in two elution modes as shown in Figure 5B. The coil is first filled with the head phase (white) followed by elution with the tail phase (black) from the head toward the tail of the coil. Alternatively, the coil is filled with the tail phase followed by elution of the head phase from the tail toward the head of the coil. In either case, the mobile phase quickly flows through the coil and is collected from the other end, leaving a large volume of the stationary phase in the coil. Consequently, solutes locally introduced at the inlet of the coil are separated in a short period of time.

The system also permits simultaneous introduction of the two solvent phases through the respective terminals of the coil to induce a true countercurrent flow of the two phases. This dual CCC system requires an additional flow tube at each end of the coil to collect the effluent and, if desired, a sample injection tube at the middle portion of the coil as shown in Figure 5C. In addition to the liquid–liquid dual CCC, this system provides a unique application to foam separation. In the foam CCC system, gas and liquid phases undergo a true countercurrent flow through a long narrow coiled tube with the aid of the Type J synchronous planetary motion. When the liquid phase contains a surfactant, the above countercurrent process produces a foaming stream that moves with the gas phase toward the tail. The sample mixture introduced at the middle of the column is separated into its components according to their foam affinity; foam active components are quickly carried with the foaming stream toward the tail whereas the remainder is carried in the liquid stream in the opposite direction and collected at the head end of the coil. For samples with a strong foaming capacity such as proteins and peptides (bacitracin), foam CCC can be performed without the use of the surfactant in the liquid phase.

In addition to the Type J planetary motion described above, some other synchronous planetary motions can produce the unilateral phase distribution (Figure 5A) that can be utilized for performing high speed CCC. Among these, the hybrid systems between Types X and L (see Figure 3) are extremely useful because they can retain a satisfactory volume of the stationary phase for viscous polymer phase systems that are used for partition of macromolecules and cell particles.

The hydrodynamic motion of the two solvent phases in the Type J high speed CCC system has been observed under stroboscopic illumination. A spiral column was filled with the stationary phase and the coloured mobile phase was eluted through the column in a suitable elution mode. After the steady-state hydrodynamic equilibrium was reached, the spiral column showed two distinct zones. As shown in the upper diagram in **Figure 6**, vigorous mixing of the two solvent phases was observed in about one quarter of the column area near the centre of the centrifuge (mixing zone), while two phases are clearly separated into two layers in the rest of the area (settling zone). Because the location of the mixing zone is fixed with respect to the centrifuge system, while the spiral column rotates about its own axis, each mixing zone is travelling through the liquid like a wave of water over the sea, as shown in the bottom diagram in Figure 6. This highlights an important fact: at any given portion of the column the two solvent phases are subjected to a repetitive partition process of alternating mixing and settling at a high frequency of over 13 times per second at 800 rpm of column rotation.

This explains the high partition efficiency attained by high speed CCC. **Figure 7** shows a typical separation of flavonoids from sea buckthorn (*Hippophae rhamnoides*) produced by the standard high speed CCC technique. Major components including isorhamnetin and quercetin are well resolved within 3 h at partition efficiencies ranging from 2000 to 3000 theoretical plates.

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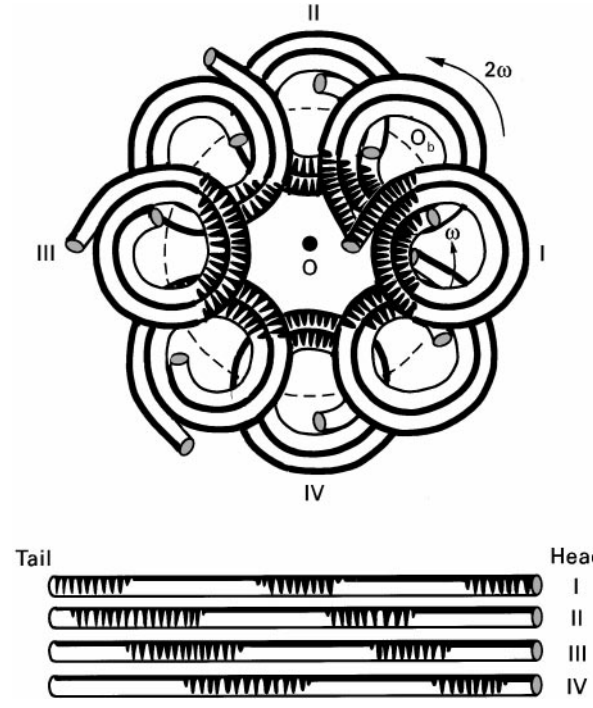

Figure 6 Hydrodynamic distribution of two solvent phases in a rotating spiral column.

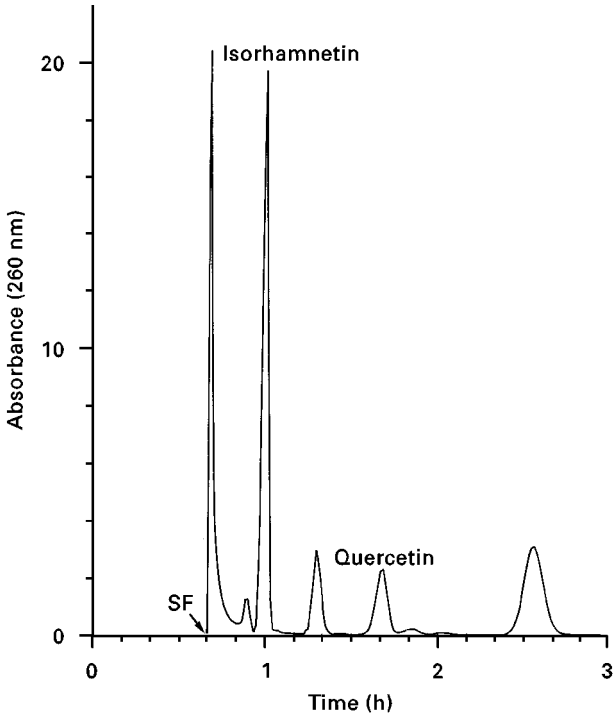

Figure 7 Separation of flavonoids from sea buckthorn by the standard high-speed CCC technique. SF, solvent front.

Standard Procedure of CCC

Selection of Two-Phase Solvent System

The first and most important step in CCC is to choose a proper solvent system that can satisfy the following requirements: the sample should be soluble and stable in the solvent system; the solvent system should provide a suitable partition coefficient for the target compounds; and it should produce a satisfactory retention of the stationary phase in the column. The partition coefficient and retention of the stationary phase are discussed below.

Partition coefficient CCC differs from other types of chromatography in that it uses two solvent phases and, therefore, the partition coefficient of the solute can be easily determined by a test tube experiment prior to the separation. In each measurement the sample is first partitioned between equilibrated two-solvent phases in a test tube, an aliquot of each phase is removed, and then the concentration of the solute in each aliquot is measured by ultraviolet or visual wavelength absorbance. Other methods of measurement such as radioactivity, enzymatic activity, etc., can be used. The partition coefficient, K, is the ratio between these two measurements and expressed in various ways such as $K(\mathrm{U/L}) = C_U/C_L$ (solute concentration in the upper phase divided by that in the lower phase), $K(\mathrm{S/M}) = C_S/C_M$ (solute concentration in the stationary phase divided by that in the mobile phase), etc. When the sample is a mixture of multiple components, the partition coefficient of each component can be obtained by high performance liquid chromatography (HPLC), gas chromatography (GC) or thin-layer chromatography (TLC) analysis of each phase and by comparing the peak height or area of the corresponding peaks in the two chromatograms. In general the most suitable range of K value is $1 < K(\mathrm{S/M}) < 2$ for the hydrostatic CCC systems and $0.5 < K(\mathrm{S/M}) < 1$ for the hydrodynamic CCC systems. Once the K value has been determined, the retention volume of the solute can be computed from the following equation:

$$V_R = V_{SF} + K(\mathrm{S/M})(V_C - V_{SF})$$

where V_R is the retention volume of the solute, V_{SF} the retention volume of the solvent front (amount of the mobile phase in the column), and V_C the total column capacity.

Retention of stationary phase The retention of the stationary phase in the separation column is an important factor in determining the resolution of solute peaks in CCC. Generally, the greater the retention of the stationary phase, the better the separation. In hydrostatic systems, in which the phase mixing is not violent, the retention of the stationary phase is conveniently adjusted by varying the flow rate of the mobile phase and/or the rotation speed in the centrifugal CCC system. In hydrodynamic systems, which provide efficient mixing of the two phases, stationary phase retention requires more careful selection of the two-solvent system as well as the choice of the mobile phase and its elution mode. In high speed CCC using the Type J planetary motion, the settling time of the two solvent phases under gravity provides a useful measure for the stationary phase retention and the elution mode. The test is performed as follows: the two phases are preequilibrated in a separatory funnel and 2 mL of each phase is delivered into a 5 mL capacity graduated cylinder equipped with a stopper (an ordinary glass test tube, 13 mm o.d. and 10 cm long with a polyethylene cap can also be used). The contents are gently mixed by inverting the container five times and the time required to form two clear layers is measured. If this settling time is within 30 s, the solvent system can be used for separation by eluting the lower phase from the head toward the tail or the upper phase in the reversed mode. If the settling time exceeds 30 s, the above elution mode should be reversed while the retention of the stationary phase is usually considerably lower than an optimum range. However, this settling time test is not

applied to the cross-axis coil planet centrifuge systems based on the planetary motions of Type X, Type L and their hybrid systems (see Figure 3, left column). These centrifuge systems provide excellent retention of the stationary phase for almost all two-phase solvent systems including viscous polymer phase systems used for partition of macromolecules and cell particles.

The retention of the stationary phase in the hydrodynamic CCC systems has been extensively studied using various two-phase solvent systems. These results are summarized in a set of phase distribution diagrams that will provide a valuable guide for users of high speed CCC systems (see Further Reading).

Preparation of the Sample Solution

In CCC, the sample solution is usually prepared by dissolving the sample in the solvents used for the separation. If the amount of sample is small, it may be dissolved in the stationary phase. However, if the sample mixture contains multiple components with a wide range of polarity, it should be dissolved in both solvent phases. In this way, the volume of the sample solution is minimized and also the two-phase formation in the sample solution is ensured. Occasionally, a single phase is formed after dissolving a large amount of the sample, which would result in a detrimental loss of the stationary phase from the column. If this occurs, the sample solution should be diluted with the solvent until two phases are formed. Although CCC permits the loading of the sample solution containing undissolved particulates, the best results are obtained by filtering the sample solution before introduction into the column.

As in other forms of LC, the amount of sample and sample solution affects the separation. Usually, a typical semipreparative column with about 300 mL capacity will separate up to a few hundred milligrams of sample dissolved in 10 mL of the solvent mixture without significantly affecting the partition efficiency.

Elution Procedure

In both hydrostatic and hydrodynamic CCC, separation is initiated by filling the entire column with the stationary phase of a mutually equilibrated two-phase solvent system. This is followed by injection of the sample solution through the sample port. Then, the mobile phase is eluted through the column in the correct elution mode while the apparatus is rotated at a suitable speed (except for DCCC). Although sample injection may be made after the column had been equilibrated with the mobile phase (the routine procedure in HPLC), this practice does not usually improve the separation in CCC.

In hydrostatic CCC systems, the direction of eluting the mobile phase should be chosen in such a way that the upper phase is eluted against the acting force field (ascending mode), and the lower phase along the force field (descending mode). In hydrodynamic CCC systems, the direction of the Archimedean screw force also plays an important role in the retention of the stationary phase. Generally speaking, the lower phase should be eluted from the head toward the tail, and vice versa for the upper phase. For various polymer phase systems (such as polyethylene glycol/dextran) with a high viscosity, maximum retention of the stationary phase is usually obtained by using the less viscous phase as the mobile phase.

As in HPLC, CCC permits the use of gradient or stepwise elution. The method requires suitable selection of the solvent system so that the volume of the stationary phase in the column is not significantly altered by the mobile phase. Some examples are *n*-butanol/water with a concentration gradient of dichloroacetic acid or trifluoroacetic acid, and polymer phase systems with a pH gradient of sodium or potassium phosphate.

Detection

In the past, detection of the CCC effluent has been performed by adapting the UV/visible absorbance monitoring system used in HPLC. In this case, a straight vertical flow cell should be used. In order to avoid trapping the droplets of the stationary phase in the cell, the mobile lower phase should be introduced from the bottom of the flow cell upward and the mobile upper phase from the top of the flow cell downward. In addition to absorbance measurement, other monitoring methods may also be used which include post-column reaction, interfacing with a mass spectrometer or nuclear magnetic resonance (NMR), laser light scattering detection, pH or conductivity measurement, etc. Most of these methods are less sensitive to the carryover of the stationary phase droplets and therefore will produce better elution curves.

Variations of Countercurrent Chromatography

The standard CCC technique described above may be modified so that it is suitable for a particular type of separation. Many of these modified CCC schemes have their counterpart in LC, utilizing a solid support matrix. **Table 1** summarizes the relationship between a variety of CCC techniques and their counterparts in LC.

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Table 1 Variations of CCC with their counterparts in LC with solid support matrix

CCC	*Description*	*Counterpart in LC*
Normal mode	Organic phase mobile	Normal-phase LC
Reversed mode	Aqueous phase mobile	Reversed-phase LC
Dual mode	Both phases mobile	Moving-bed LC
Ion CCC	Ion exchanger in SP[a]	Ion LC
Affinity CCC	Affinity ligand in SP	Affinity LC
pH-Zone-refining CCC	Retainer in SP; eluter in MP[b]	Displacement LC
Chiral CCC	Chiral selector in SP	Chiral LC

[a]SP, stationary phase.
[b]MP, mobile phase.

Ion CCC

Analogous to ion chromatography, CCC can separate both inorganic and organic ions according to their pK_a of affinity to the ionic ligand dissolved in the stationary phase. One example is illustrated in **Figure 8**, which shows separation of rare earth elements by pH gradient elution of a hydrochloric acid mobile phase through a hexane stationary phase containing a ligand, di-(2-ethylhexyl)phosphoric acid. **Figure 9** is another example showing the separation of catecholamine. The left chromatogram, obtained with a ligand-free solvent system, shows no sign of separation (Figure 9A). When a basic ligand, triethylamine, is added to the stationary phase, all components are resolved (Figure 9B).

Chiral Countercurrent Chromatography

Enantiomers may be resolved by CCC if an appropriate chiral selector is present in the stationary phase. **Figure 10** shows the separation of four enantiomeric pairs of dinitrobenzoyl amino acids using a chiral selector, *N*-dodecanoyl-L-proline-3,5-dimethylanilide in the stationary phase. When the separation is performed without the chiral selector, all components elute near the solvent front, resulting in poor separation (Figure 10A). The addition of the chiral selector to the stationary phase results in excellent separation with only one overlapping peak under otherwise identical experimental conditions (Figure 10B). Gram quantities of ionic enantiomers can be purified by means of the pH-zone-refining CCC technique described below.

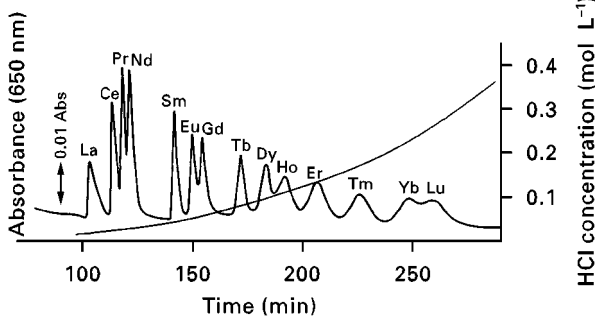

Figure 8 Analysis of rare earth elements by pH gradient elution with a ligand in the stationary phase. Apparatus, HSCCC centrifuge with 7.5 cm revolution radius; column, three multilayer coils, 1.1 mm i.d. × 300 m, 270 mL capacity; stationary phase, 0.003 mol L^{-1} di-(2-ethylhexyl)phosphoric acid in *n*-heptane; mobile phase, exponential gradient of 0–0.4 mol L^{-1} HCl; sample, 0.001 mol L^{-1} each of La, Ce, Pr, Nd, Sm, Eu, Gd, Tb, Dy, Ho, Er, Tm, Yb and Lu in 100 μL; revolution, 900 rev min^{-1}; flow rate, 5 mL min^{-1}; pressure, 300 psi (~ 2070 kPa).

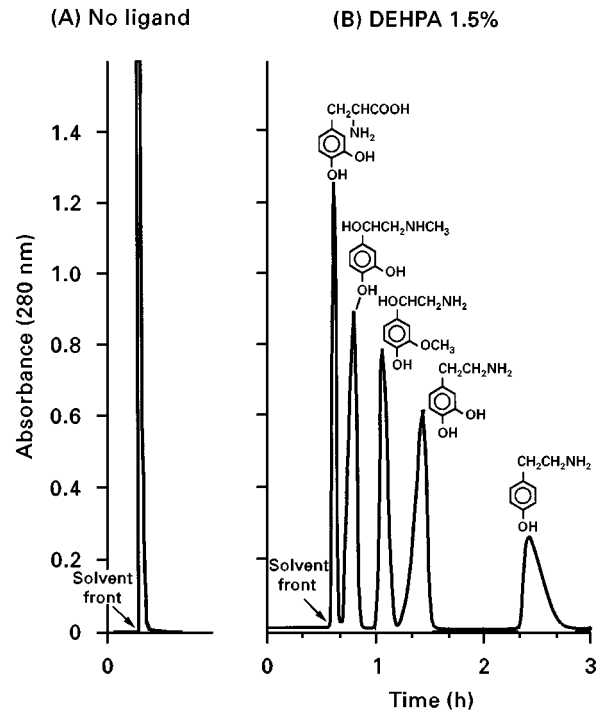

Figure 9 Separation of catecholamine and related compounds by high speed CCC using a ligand, triethylamine, in the stationary phase. The left chromatogram (A) obtained without ligand shows no evidence of separation. All components were completely resolved by introducing the ligand in the stationary phase, as shown in part (B).

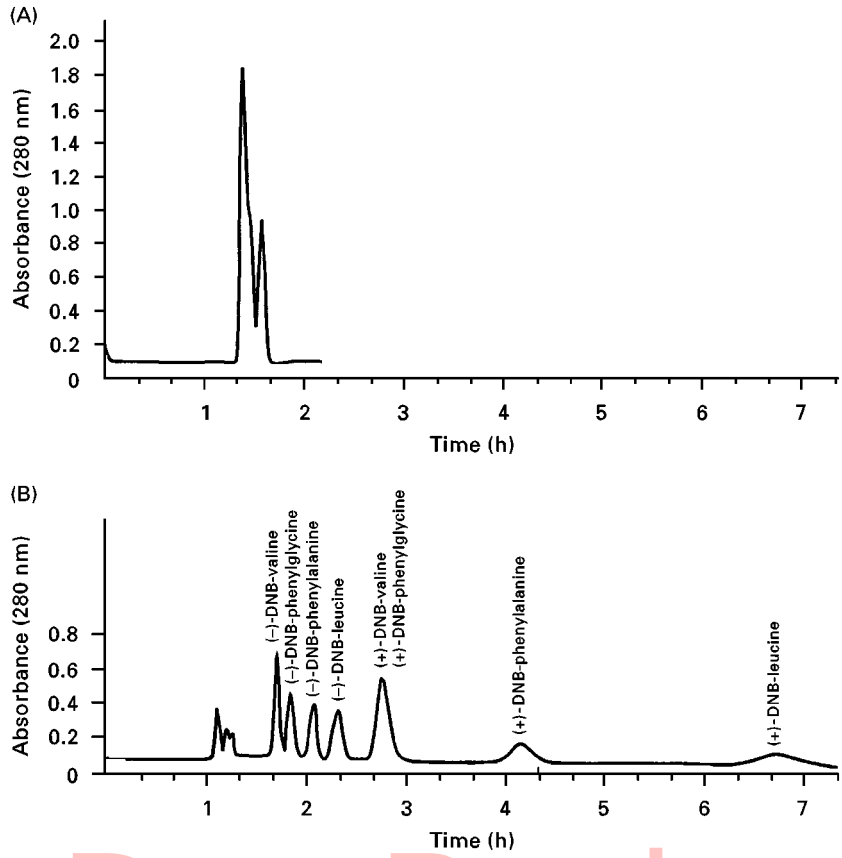

Figure 10 Separation of four enantiomeric dinitrobenzoyl (DNB) amino acids by high speed CCC, with (B), and without (A), a chiral selector, *N*-dodecanoyl-L-proline-3,5-dimethylanilide (DPA). In the upper chromatogram obtained without chiral selector, all components eluted near the solvent front with poor peak resolution. Introduction of 1.6 g of DPA in the stationary phase under otherwise identical experimental conditions resulted in a remarkable improvement in peak resolution.

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pH-Zone-Refining CCC

This preparative CCC technique produces a train of highly concentrated rectangular peaks similar to those obtained in displacement chromatography. The method utilizes a retainer acid or base in the stationary phase and an eluter counterion in the mobile phase. Interaction of ionic analytes forms a series of solute zones with sharp boundaries that move together through the column at the same rate (isotachic movement). Each zone consists of a single species, has its own specific pH and is arranged in the order of its pK_a and hydrophobicity. Charged minor components are concentrated at the boundaries of the major zone

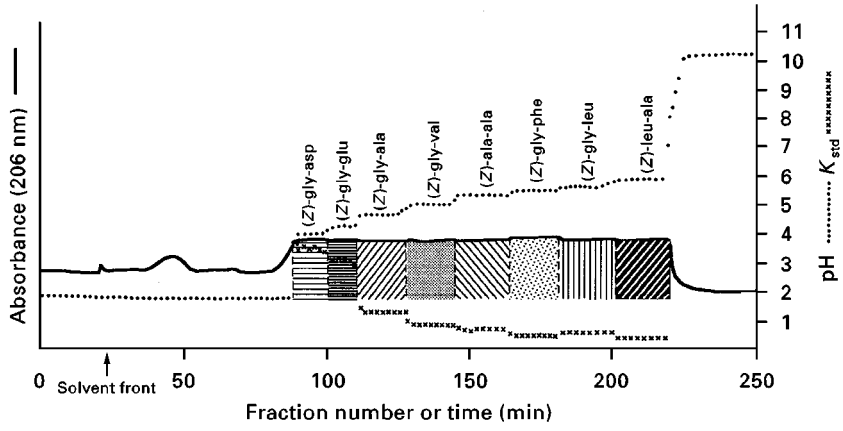

Figure 11 Separation of eight (*Z*)-(benzyloxycarbonyl) dipeptides by pH-zone-refining CCC.

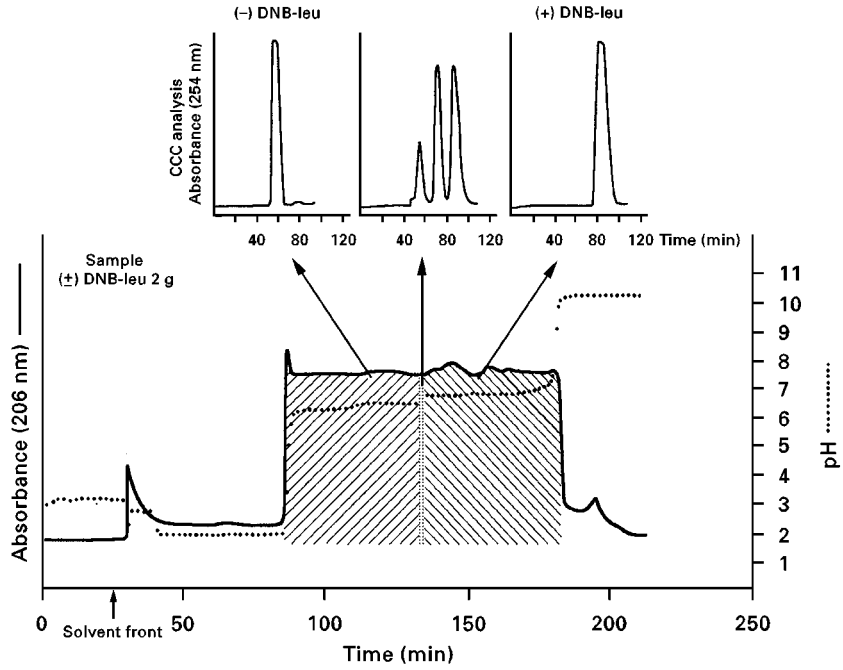

Figure 12 Separation of (±)-dinitrobenzoyl leucine ((±) DNB-leu) by pH-zone-refining CCC.

and are eluted as sharp peaks. **Figure 11** shows the separation of eight (*Z*)-(benzyl-oxycarbonyl) dipeptides by pH-zone-refining CCC. All components (100 mg of each) are well resolved with minimum mixing zones as evidenced by the sharp transitions of their partition coefficients (K_{std}) measured with a standard two-phase solvent system.

The method can also be applied to preparative-scale chiral separation using a chiral selector in the stationary phase. **Figure 12** illustrates separation of 2 g of (±)-dinitrobenzoyl leucine by pH-zone-refining CCC. pH-zone-refining CCC is described in detail in texts listed in Further Reading.

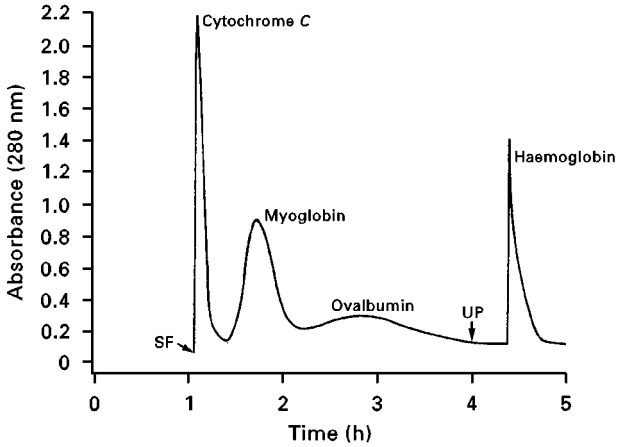

Figure 13 Protein separation with an aqueous–aqueous polymer phase system composed of 12.5% (w/w) polyethylene glycol 1000 and 12.5% (w/w) K_2HPO_4 in distilled water. The column was initially eluted with the lower phase. After point UP the column was eluted with the upper phase in the reverse direction to elute haemoglobin retained in the column SF, solvent front.

CCC with Polymer Phase Systems

Separation of proteins requires the use of aqueous–aqueous polymer phase systems to prevent denaturation of the analytes by organic solvents. **Figure 13** shows separation of four stable proteins by a polymer phase system composed of 12.5% (w/w) polyethylene glycol 1000 and 12.5% (w/w) dibasic potassium phosphate. The separation was performed by a cross-axis coil planet centrifuge (hybrid between Type L and X in Figure 2). The method has also been successfully applied for purification of recombinant enzymes.

Conclusions

Countercurrent chromatography covers a broad spectrum of samples ranging from small ions to macromolecules. The method provides various advantages over conventional liquid chromatographic techniques such as no sample loss and denaturation due to the solid support, high purity of fractions and high reproducibility. The CCC technique is particularly suitable for preparative separations of natural and synthetic compounds.

See also: **II/Chromatography:** Countercurrent Chromatography and High-Speed Countercurrent Chromatography:

Instrumentation. **Chromatography: Liquid:** Partition Chromatography (Liquid–Liquid). **III/Chiral Separations:** Countercurrent Chromatography; Liquid Chromatography. **Foam Countercurrent Chromatography. Ion Analysis:** Liquid Chromatography; High-Speed Countercurrent Chromatography. **Liquid Chromatography. Medicinal Herb Compounds: High-Speed Countercurrent Chromatography. Natural Products:** High-Speed Countercurrent Chromatography; Liquid Chromatography. **Peptides and Proteins:** Liquid Chromatography. **Proteins:** High-Speed Countercurrent Chromatography.

Further Reading

Conway WD (1990) *Countercurrent Chromatography: Apparatus, Theory and Applications*. New York: VCH.

Conway WD and Petroski RJ (eds) (1995) *ACS Symposium Series Monograph on Modern Countercurrent Chromatography*. Washington, DC: American Chemical Society.

Ito Y (1981) Countercurrent chromatography (minireview). *Journal of Biochemical and Biophysical Methods* 5: 105–129.

Ito Y (1986) High-speed countercurrent chromatography. *CRC Critical Reviews in Analytical Chemistry* 17: 65–143.

Ito Y (1991) Countercurrent chromatography in Chromatography V, part A, chap. 2, pp. A69–A107. Amsterdam: Elsevier.

Ito Y (1996) Countercurrent chromatography. In: *Encyclopedia of Analytical Science*, pp. 910–916. London: Academic Press.

Ito Y (1996) pH-zone-refining countercurrent chromatography. *Journal of Chromatography A* 753: 1–30.

Ito Y and Conway WD (eds) (1996) *High-Speed Countercurrent Chromatography*. New York: Wiley-Interscience.

Mandava NB and Ito Y (1988) *Countercurrent Chromatography: Theory and Practice*. New York: Marcel Dekker.

Derivatization

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Introduction

Derivatization involves changing in some way the basic chemical or physical structure of a compound, usually to a single product, which may be more useful for the analysis of the original analyte in liquid chromatography (LC). Derivatization can be used for analytical or preparative scale LC. In the analytical mode, it can be used to improve the identification and quantitation of the analyte of interest. It may also be used to improve throughput and recovery in preparative scale LC purifications of large amounts of material. Changes in the basic structure of the analyte can also lead to improved peak shape, elution times, peak symmetry, efficiency, plate count, and other indicators of chromatographic performance. That is, elution times and retention factors, as well as resolution, separation factors, reduced plate heights, and other LC parameters of performance, can all be varied and improved by suitable, selective derivatization of the starting analyte.

The most general type of derivatization involves modifying the chemical structure of the starting compound by tagging or adding another reagent to it via a suitable functional group alteration (**Figure 1**). Thus, most simple derivatizations involve a derivatizing reagent, the substrate or analyte of interest, the desired derivative of the analyte, remaining excess reagent, and undesirable by-products coming from the excess derivatizing reagent reacting with solvent, water or thermally degrading (Figure 1). Ideally, only the desired derivative would remain at the end of the reaction period, without any remaining starting analyte, derivatizing reagent or by-products. However, this idealized situation is rarely observed and it is often necessary to separate prior to or during the LC analysis the desired derivative from all other possible compounds coming from the derivatization reaction and/or sample components and their possible derivatization products.

Though most derivatizations usually occur in a homogeneous solution between the analyte of interest and the reagent itself, it is possible to perform derivatizations on the analyte in solution with an immobilized or solid-phase reagent. **Figure 2** illustrates a typical immobilized or solid-phase reagent that has been described in the literature for use with LC. It is also feasible to first immobilize the analyte on a solid support, such as silica gel, Immobilon™ membrane, poly(styrene-divinylbenzene), C_{18} packing material, and others, and then perform the derivatization reaction on the now-immobilized analyte. Once the reaction is completed, the excess reagent is simply washed from the solid support still containing the derivative. The desired derivative is then eluted with a stronger solvent from the solid support, often in a disposable plastic tube (solid-phase extraction cartridge or Sep-Pak™), without any residual, unreacted starting